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Allelic loss on chromosome 13q14 and mutation in deleted in cancer 1 gene in esophageal squamous cell carcinoma

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Previous studies have shown frequent allelic loss on chromosome 13 in esophageal squamous cell carcinoma (ESCC). We assessed the frequency of allelic loss on chromosome 13q14 and mutations of deleted in cancer 1 (*DICE1*) (also found on 13q14) in ESCC patients to determine if this candidate tumor suppressor gene has a role in the development of ESCC, and whether this gene was an inactivation target for allelic loss on chromosome 13q14. Initially, we examined allelic loss at five markers flanking *DICE1* in 56 ESCC patients from Shanxi Province, China, and then examined the entire coding sequence of this gene for mutations using polymerase chain reaction–single-strand confirmation polymorphism (PCR–SSCP) analysis and DNA sequencing. Subsequently, we extended our evaluation to an additional 80 ESCC patients and 232 healthy individuals to confirm the germline variant found in the initial 56 ESCC patients. The frequencies of allelic loss were 71, 58, and 75% for D13S325, D13S757, and D13S887, respectively, in the initial 56 ESCC patients studied. Overall, 73% of informative patients had loss of heterozygosity (LOH) for at least one of these three markers. Somatic mutations were identified in three patients (3/56, 5%), and one novel polymorphism was identified in 3% of ESCC patients (4/136) and 3% of healthy individuals (6/232). We conclude that *DICE1* mutations occur in ESCC but are infrequent. The candidate tumor suppressor gene corresponding to the frequent allelic loss on chromosome 13q14 in ESCC remains unknown.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is very common in many areas of China, especially in Shanxi Province (Hu *et al*., 1992). In previous studies in Shanxi Province, we found frequent loss of heterozygosity (LOH) on chromosome 13q (Hu *et al*., 1999, 2000). Although LOH on chromosome 13q is frequently detected in many types of tumors (Kuroki *et al*., 1995; Montesano *et al*., 1996; Eiriksdottir *et al*., 1998; Hyytinen *et al*., 1999), only two tumor suppressor genes have been identified on this chromosome to date, *RB1* (13q14) and *BRCA2* (13q12) (Lee *et al*., 1987; MacGee *et al*., 1989; Wooster *et al*., 1995). LOH at the *RB1* locus has been reported in 54% of ESCCs (Boynton *et al*., 1991; Huang *et al*., 1992), but few mutations have been detected (Maesawa *et al*., 1994). We reported frequent allelic loss on chromosome 13q12 (Li *et al*., 2001) in ESCC patients, but infrequent detection of mutations in *BRCA2* (Hu *et al*., 2002). All these studies suggest that other as yet unknown gene(s) on chromosome 13q may be involved in the pathogenesis of ESCC. A candidate tumor suppressor gene, deleted in cancer *1* (*DICE1*) (GenBank database Accession AF097645) was recently isolated and localized to chromosome 13q14.12–q14.2 (Wieland *et al*., 1999). *DICE1* has 887 amino acids, is highly conserved in evolution, and its mRNA is expressed in a wide variety of fetal and adult tissues. *DICE1* corresponds to an evolutionarily highly conserved protein of 100 kDa suggesting an important cellular function (Wieland *et al*., 1999). In addition, *DICE1* showed loss or downregulation of expression in most nonsmall cell lung carcinomas tested (Wieland *et al*., 1999). Although *DICE1* is located in a critical region where LOH is commonly observed in many types of human cancers, it has not yet been studied in many human cancers, including esophageal cancer.

In the present study, we examined LOH on chromosome 13q14 using five markers flanking *DICE1* in ESCC patients from Shanxi, China, and then tested for mutations in this gene using single-strand confirmation polymorphism (SSCP) analysis and DNA sequencing in the same group of patients.

Results

Allelic loss in ESCC patients

We found that all 56 ESCC patients we initially tested were homozygous for markers D13S1203 and

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D13S1161. For the remaining three markers, D13S325, D13S757, and D13S887, the frequencies of LOH were 71% (24 of 34 informative cases), 58% (15 of 26 informative cases), and 75% (nine of 12 informative cases), respectively. Overall, 73% (34 of 44 cases informative at one or more of these three markers) had LOH for at least one marker.

Alterations of DICE1 in ESCC patients

Screening the entire coding region of *DICE1* in tumor and blood DNA of 56 ESCC patients identified somatic mutations in three patients (3/56, 5%) and a 3 bp intronic germline deletion in two cases. These alterations are listed in Table 1 and examples are shown in Figures 1 and 2.

Table 1 LOH on 13q14, *DICE1* alterations, and descriptors of five ESCC patients

Experimental or clinical data		*Patient ID*				
		SHE057	*SHE096*	*SHE208*	*SHE273*	*SHE480*
Description of alteration	Variant	Somatic	Somatic	Germline	Germline	Somatic
	Exon	11	11	Intron 1	Intron 1	7
	Codon	658	658	Nucleotide 6033–6035[a]	Nucleotide 6033–6035	431
	Base change	g→a	c→a	3 bp del ctt(ctt)tttttaat	3 bp del ctt(ctt)tttttaat	g→a
	Amino-acid change	cgg→cag Arg→Gln	cgg→agg Arg→Arg	—	—	gtt→att Val→Ile
	Designation	R658Q (missense mutation)	R658Q (missense mutation)	6033–6035delCTT	6033–6035delCTT	V431V (missense mutation)
	Intragenic allelic loss	No	No	Lost variant allele	Lost variant allele	Lost wildtype allele
LOH	13S325	NI	R	NI	LOH	LOH
	13S757	NI	NI	NI	LOH	R
	13S887	NI	NI	NI	NI	NI
Evidence for biallelic alterations		No	No	No	No	Yes
Demographic, clinical/path characteristics, cancer lifestyle risk factors	Age/sex	50/F	57/M	56/F	62/M	65/M
	Location of tumor	Middle	Middle	Middle	Middle	Lower
	Grade	2	1	2	2	1
	Stage	III	III	III	III	III
	Lymph node metastasis	Yes	No	No	Yes	Yes
	Smoker	No	Yes	Yes	No	Yes
	Drinks alcohol	Yes	Yes	No	Yes	Yes
	Eats pickled vegetables	No	Yes	Yes	Yes	Yes
	Eats scaling hot food	No	Yes	Yes	Yes	Yes
	Family history of cancer	EC (sister)	No	No	No	EC (paternal uncle)

[a]http://www.ncbi.nlm.nih.gov/, locus no.12738222.
NI: not informative; R: retention, EC: esophageal cancer.

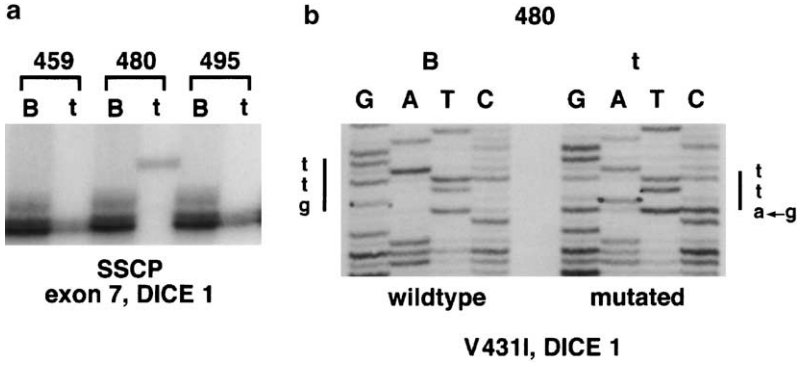

Figure 1 Somatic mutation of *DICE1* in case #480. (**a**) SSCP gel shows an abnormal migration pattern in the tumor. (**b**) Sequencing gel shows a missense mutation, g→a, resulting in an amino-acid change of Val→Ile at codon 431 (V431I)

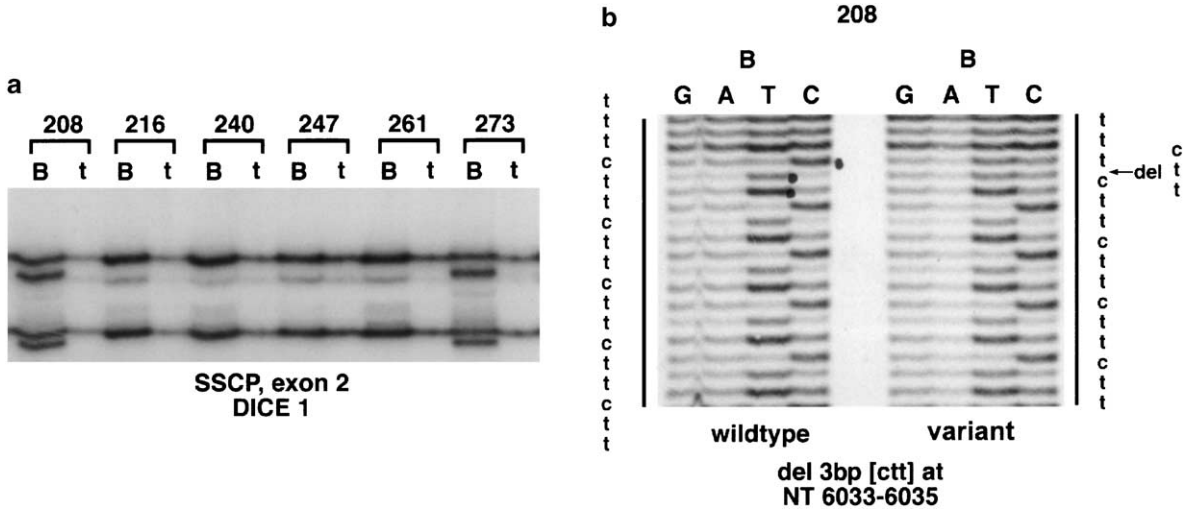

Figure 2 Germline alteration of *DICE1* in cases #208 and #273. (**a**) SSCP gel shows abnormal migration pattern in germline DNA; sequencing result demonstrates that bands one and three are strands of the wild-type allele and bands two and four are strands of the variant allele in germline DNA. (**b**) Sequencing gel shows a germline alteration 6033–6035delCTT

Demographic and clinical/pathological characteristics as well as selected cancer lifestyle risk factors for these five patients with a *DICE1* alteration are listed in Table 1. We found evidence for potential biallelic alteration of *DICE1* in just one of 56 ESCC (2%) cases (Table 1). This case (#480) had a missense mutation on one allele and lost the wild-type allele in tumor.

A novel polymorphism in Chinese

To evaluate whether the germline variant (6033–6035del CTT) was a specific germline mutation in ESCC or a novel polymorphism in Chinese, we examined an additional set of ESCC patients as well as healthy individuals. The germline variant was observed in two of 80 additional ESCC patients, five of 101 healthy individuals from Beijing, and one of 131 healthy individuals from Yangcheng. The overall variant frequencies were 3% (4/136) in ESCC patients and 3% in healthy individuals (6/232).

Genetic alterations of DICE1 and LOH on 13q14

The number of cases with a *DICE1* mutation was too small for meaningful comparison with LOH at 13q14. Of 34 informative cases at D13S325, two (2/24, 8%) had a mutation in *DICE1*; one of 15 (7%) informative cases at D13S757 had a mutation; and none of 12 informative cases at D13S887 had a mutation in this gene.

Discussion

Mutation in *DICE1* has been previously studied only in nonsmall cell lung cancer, where Wieland *et al*. reported loss or downregulation of expression (Wieland *et al*., 1999). Ours is the first study of *DICE1* in esophageal cancer and the first to identify germline or somatic alterations in *DICE1* in any cancer. We found three ESCC patients (5%) with *DICE1* mutations. In addition, two of the initial 56 ESCC patients we tested had the same germline variant (6033–6035delCTT). To determine whether this variant was a specific germline mutation of *DICE1* or a novel polymorphism in Chinese, we tested more ESCC patients as well as healthy individuals from Beijing (a low-risk area for ESCC) and Yangcheng County (a high-risk area for ESCC), and found a frequency of 3% in both ESCC and healthy individuals, suggesting that this germline variant is a novel polymorphism in the Chinese population. Among the cases evaluated, only one (#480) showed evidence for potential biallelic alterations in *DICE1*. This patient had a missense mutation at codon 431, part of the most conserved region (codon 114–444) of *DICE1* gene product (Wieland *et al*., 1999), and lost the wild-type allele. We do not know, however, if function was altered in this case. Our result indicated only that mutation of *DICE1* is an infrequent event. Epigenetic events affecting transcription, however, may occur and remain to be evaluated (Wieland *et al*., 2001).

While allelic loss on 13q14 was observed frequently in our patients, most of the patients who had LOH in this region did not have a mutation in *DICE1*. Of the five cases who had a mutation in this gene, two had LOH, two were homozygous for all three markers flanking this gene, and one showed retention at one marker and homozygosity at the other two markers. The modest number of ESCC cases evaluated here does not permit definitive conclusions regarding the role of mutations in *DICE1* and LOH on 13q14; however, *DICE1* does not appear to be the target gene affected by LOH on chromosome 13q14. Thus, the gene corresponding to the frequent allelic loss on chromosome 13q14 remains unknown.

Table 2 Sequence of primers used for PCR–SSCP analysis of *DICE1*

Exon	*Sense primer (5′–3′)*	*Antisense primer (5′–3′)*	*PCR product size (bp)*	*Anneal temperature (°C)*
2	AGTTGTTTTTTTCTTGCTAA	GAATAAACAATAAAGCAAGT	270	48
3	AACTTTTTATGCTTATCCC	ATTACATAGTTACAGTCTCT	247	48
4	AGACTTTCCCCTTTAGCC	TACATAAACCATTGATTC	250	50
5	GAATCAATGGTTTATGTA	AGTGTTTCAATATCATCTTT	390	46
6	AAATGACAAAACCGTAAATC	TGGGATTACAGGCGTGAGTC	315	55
7	ATACGATTGTGTTACTTCCT	ATGGCTCACTCTATTTCTTG	274	50
8	ATTCTGACTTATTTTTACTT	AACATAACAGAACATAACT	286	46
9	AGTCTCTTGTAATTCTATGT	TGCTATGCTTTTCAGACTAT	250	43
10	AACATTATTTGAGAACTA	GTGCCTACCATAAACAAAT	248	52
11	AGTTGTATTCTTTTTTGCTA	AAAGGCAGCAAATAATGTT	295	46
12.1	TTTCCCCGTATCTTTGTTT	TTCTAAAAATCCACCAACAG	235	52
12.2	TGACCATTTAGGAACCAA	AGCACATACAGAAATCAGA	253	46
13	TGAATAAGAATACAAATGAA	TTCATCACCATTTCATTAT	189	46
14	TACTATTTATTTTTTCAT	AGAAGATAGTGAAATAAGTG	154	46

Materials and methods

Patient selection

Patients presenting in 1995 and 1996 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, China, who were diagnosed with ESCC and considered candidates for curative surgical resection, were identified and recruited to participate in this study. This research study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the US National Cancer Institute (NCI). A total of 56 patients with ESCC who had a histologic diagnosis of ESCC confirmed by pathologists at both the Shanxi Cancer Hospital and the NCI were selected. None of the patients had prior therapy, and Shanxi was the ancestral home for all.

After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors, and a detailed family history of cancer. A total of 56 ESCC patients, including 34 males and 22 females, were evaluated. Details on these patients have been previously reported (Huang *et al*., 2000).

An additional second set of 80 ESCC patients from the Shanxi Cancer Hospital (who presented in the same manner as the 56 cases described above) and 232 healthy individuals from blood banks (101 from Beijing and 131 from Yangcheng, Shanxi Province) were examined for a germline variant that was found during the examination of the initial 56 ESCC patients in the present study.

Biologic specimen collection and processing

Venous blood (10 ml) was taken from each patient prior to surgery. Blood from healthy individuals was collected from blood banks in Beijing and Yangcheng, China. Genomic DNA was extracted and purified. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser microdissection and extraction of DNA

Tumor cells were microdissected under light microscopic visualization using methods previously described (Emmert-Buck *et al*. 1996; Huang *et al*., 2000).

Markers, polymerase chain reaction (PCR), and LOH reading and interpretation

Five microsatellite markers, D13S325 (13q14.11), D13S1203 (13q14.12), D13S757 (13q14.2), D13S887 (13q14.2), and D13S1161 (13q14.2), were selected (Human MapPairs™ Research Genetics, Huntsville, AL, USA). The total distance from D13S325 to D13S1161 is estimated at 8 Mb (http://cedar.genetics.soton.ac.uk/public_html/read.html). *DICE1* is located between D13S1203 and D13S1757. DNA extracted from tumor cells was microdissected from resection specimens, and genomic DNA extracted from venous blood was used for each patient. PCR reactions were carried out as previously reported (Huang *et al*., 2000). LOH was defined as either complete or nearly complete loss of a band in the tumor sample relative to the corresponding normal DNA.

PCR, SSCP analysis, and DNA sequencing

Mutations in all 13 coding exons of *DICE1* were screened by PCR–SSCP. The 14 pairs of PCR primers used to cover all intron/exon boundaries are listed in Table 2. Tumor and normal DNA were used for each patient. PCR reactions, SSCP analyses, and DNA sequencing were conducted using methods previously described (Hu *et al*., 2001). The annealing temperatures for each PCR reaction are listed in Table 2. All SSCP gels with band shifts were repeated and are reported as positive only when the shifts were confirmed.

Abbreviation:
ESCC, esophageal squamous cell carcinoma; SSCP, single-strand confirmation polymorphism; PCR, polymerase chain reaction; LOH, loss of heterozygosity.

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